Protein ingestion induces muscle insulin resistance independent of leucine-mediated mTOR activation

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ABSTRACT

Increased plasma branched-chain amino acid concentrations are associated with insulin resistance and intravenous amino acid infusion blunts insulin-mediated glucose disposal. We tested the hypothesis that protein ingestion impairs insulin-mediated glucose disposal by leucine-mediated mTOR signaling, which can inhibit AKT. We measured glucose disposal and muscle p-mTOR<sub>Ser2448</sub>, p-AKT<sup>Ser473</sup> and p-AKT<sup>Thr308</sup> in 22 women during a hyperinsulinemic-euglycemic clamp procedure with and without concomitant ingestion of whey protein (0.6 g per kg fat-free mass; n=11) or leucine that matched the amount given with whey protein (n=11). Both whey protein and leucine ingestion raised plasma leucine concentration by ~2-fold and muscle p-mTOR<sub>Ser2448</sub> by ~30% above the values observed in the control (no amino acid ingestion) studies; p-AKT<sup>Ser473</sup> and p-AKT<sup>Thr308</sup> were not affected by whey protein or leucine ingestion. Whey protein ingestion decreased insulin-mediated glucose disposal (38.8 [30.8, 61.8] vs 51.9 [41.0, 77.3] µmol glucose per µU insulin·ml<sup>-1</sup>·min<sup>-1</sup>; P<0.01; medians [quartiles]) whereas ingestion of leucine did not (52.3 [43.3, 65.4] vs 52.3 [43.9, 73.2]). These results indicate that: 1) protein ingestion causes insulin resistance and could be an important regulator of postprandial glucose homeostasis, and 2) the insulin-desensitizing effect of protein ingestion is not due to inhibition of AKT by leucine-mediated mTOR signaling.

CLINICAL TRIAL REGISTRATION

The studies presented in this manuscript are registered as trial numbers NCT01538836 and NCT01757340 in Clinical Trials.gov.
Skeletal muscle insulin resistance is a common metabolic complication of obesity and is the key factor responsible for abnormal postprandial glucose clearance and increased risk for developing type 2 diabetes and cardiovascular disease in obese people (1-3). It has been suggested that branched-chain amino acids (i.e., leucine, isoleucine, and valine) (4; 5), most likely leucine alone (6-8), are involved in the pathogenesis of obesity-associated insulin resistance because: 1) branched-chain amino acid concentrations in plasma and their metabolites are increased in obese compared with lean people (9; 10) and have been identified as predictors of insulin resistance (9; 11-14); 2) data from studies conducted in cultured myotubes and isolated rat skeletal muscles have demonstrated that leucine can impair insulin-mediated glucose uptake (15; 16), presumably via AMPK-mediated mTOR-p70S6K phosphorylation and subsequent serine phosphorylation of IRS-1 (7; 15-19), and 3) infusing amino acids during a hyperinsulinemic-euglycemic clamp procedure can reduce glucose disposal in people (20-23). Collectively, these data suggest that dietary protein (or leucine) ingestion might be an important regulator of muscle insulin sensitivity, but we are unaware of any studies that evaluated this issue. A better understanding of the interaction between dietary amino acid availability and insulin-mediated muscle glucose uptake could help elucidate the mechanisms responsible for obesity-associated abnormalities in glucose metabolism.

The goal of the present study was to test the hypothesis that protein ingestion impairs insulin-stimulated glucose disposal due to leucine-mediated mTOR phosphorylation in muscle. Accordingly, we predicted that both whey protein ingestion and ingestion of leucine that matches the whey protein leucine content would impair insulin-mediated glucose disposal and be associated with decreased p-AMPK$^{Thr172}$ (and its downstream target p-ACC$^{Ser79}$), increased p-mTOR$^{Ser2448}$ (and its downstream target p-p70S6K$^{Thr389}$), and decreased p-AKT$^{Ser473}$ and p-
AKT\textsuperscript{Thr308} (and their downstream target GSK\textsubscript{β}\textsuperscript{Ser9}) in skeletal muscle. To accomplish this goal, two groups of subjects completed two hyperinsulinemic-euglycemic clamp procedures: one with and another without simultaneous whey protein ingestion or one with and another without simultaneous ingestion of leucine that matched the amount present in whey protein. Furthermore, we selected a dose of whey protein (and leucine) that would elicit a rise in plasma leucine concentration similar to that observed after mixed meal ingestion (24; 25).

**RESEARCH DESIGN AND METHODS**

**Subjects and pre-study testing**

Twenty-two sedentary (<1.5 hour of exercise/week) and weight-stable (<2 kg change for at least 6 months), 50-65 year old (mean ± SD: 57.8 ± 4.2 y) postmenopausal women participated in this study, which was approved by the Institutional Review Board of Washington University School of Medicine in St. Louis, MO. Written informed consent was obtained from all subjects before participation. All subjects completed a history and physical examination, a resting electrocardiogram, standard blood tests, and an oral glucose tolerance test. None of the subjects had evidence of chronic illness or significant organ dysfunction (e.g., diabetes mellitus, liver cirrhosis), or were taking medications (including hormone replacement therapy) that could interfere with insulin or glucose metabolism, and none reported excessive alcohol intake or consumed tobacco products. Body fat mass and fat-free mass (FFM) were determined by using dual energy X-ray absorptiometry (DXA, Lunar iDXA, GE Healthcare Lunar, Madison, WI). Intra-abdominal and abdominal subcutaneous adipose tissue volumes were quantified by using magnetic resonance imaging (1.5-T superconducting magnet; Siemens, Iselin, NJ) and Matlab.
software (Mathworks, Natick, MA) in the Washington University School of Medicine Center for Clinical Imaging Research.

Experimental design

Each subject completed two hyperinsulinemic-euglycemic clamp procedures and was randomized to clamp procedures conducted with or without simultaneous whey protein ingestion (n=11) or clamp procedures conducted with or without simultaneous leucine ingestion (n=11) (Table 1). Before each clamp procedure, subjects were instructed to adhere to their usual diet and to refrain from vigorous physical activities for three days. Subjects were admitted to the Clinical Research Unit in the late afternoon, where they consumed a standard dinner between 1800 and 1900 h, and then fasted, except for water, until the next morning. At 0600 h, a catheter was inserted into an arm vein for the infusion of a stable isotope labeled glucose tracer; catheters for blood sampling were inserted into the radial artery of the opposite arm and in retrograde fashion into the femoral vein of one leg. At ~0645 h, a primed, constant infusion of [\textsuperscript{6,6-}\textsuperscript{2}H\textsubscript{2}]glucose (priming dose: 22 µmol·kg body wt\textsuperscript{-1}, infusion rate: 0.22 µmol·kg body wt\textsuperscript{-1}·min\textsuperscript{-1}), purchased from Cambridge Isotope Laboratories Inc. (Andover, MA), was started and 4 h later the hyperinsulinemic-euglycemic clamp procedure was initiated with two 5-minute priming doses (first 200 mU·m\textsuperscript{2} body surface area (BSA)·min\textsuperscript{-1} then 100 mU·m\textsuperscript{2} BSA·min\textsuperscript{-1}) of human insulin (Novolin R, Novo Nordisk, Princeton, NJ); for the remaining 230 min, insulin was infused at a rate of 50 mU·m\textsuperscript{2} BSA·min\textsuperscript{-1}. Euglycemia (at blood glucose concentration ~5.6 mM) was maintained by variable rate infusion of 20% dextrose (Baxter, Deerfield, IL) enriched to 2.5% with [\textsuperscript{6,6-}\textsuperscript{2}H\textsubscript{2}]glucose. Subjects in the whey protein trial consumed either 0.6 g of whey protein (unflavored Unjury\textsuperscript{®}, ProSynthesis Laboratories, Inc, Reston, VA) per kg FFM...
(containing 0.0684 g leucine per kg FFM), dissolved in 360 ml water or the same volume of water alone during the clamp procedure. In the leucine trial, subjects consumed either 0.0684 g leucine (Sigma-Aldrich, Inc, St Louis, MO) per kg FFM, dissolved in 360 ml Kool-Aid® (Kraft Foods, Inc, Northfield, IL) or the same volume of the Kool-Aid® solution alone during the clamp procedure. To minimize potential differences in plasma leucine concentration between studies that could arise from differences in the intestinal absorption rate of free compared with whey protein-derived leucine (26; 27) and to elicit a rise in plasma leucine concentration similar to that after mixed meal ingestion (i.e., sustained approximate doubling for ~3-4 h) (24; 25) after both whey protein and leucine ingestion, both the whey protein and leucine drinks were consumed in small aliquots every 20 min. The whey/no-whey and leucine/no-leucine studies were conducted in randomized order 1-4 weeks apart.

Blood samples to determine plasma glucose, insulin and leucine concentrations and glucose kinetics were obtained immediately before starting the glucose tracer infusion and every 6-7 min during the last 20 min of the basal period and the clamp procedure; additional blood samples were obtained every 10 min during the clamp procedure to monitor blood glucose concentration. Muscle tissue from the quadriceps femoris was obtained from 9 of 11 subjects in the whey protein group and 8 of 11 subjects in the leucine group by using a Tilley-Henkel forceps 60 min after starting the glucose tracer infusion (basal period) and 180 min after starting the insulin infusion to determine the contents of p-AMPK^{Thr172}, p-ACC^{Ser79}, p-mTOR^{Ser2448}, p-p70S6K^{Thr389}, p-AKT^{Ser473}, p-AKT^{Thr308}, and GSKβ^{Ser9}. The basal and clamp biopsies were taken through separate incisions (~5 cm apart) on the same leg.

Leg blood flow in the common femoral artery was measured between 120 and 180 min after starting the glucose tracer infusion (basal period) and between 60 and 180 min after starting the
insulin infusion (clamp period) by using Doppler ultrasound (M-Turbo; Sonosite Inc, Bothell, WA) and a linear array 13 to 6 MHz frequency probe (Sonosite Inc, Bothell, WA) (28).

Sample collection, processing and analyses

Blood samples were collected in chilled tubes containing heparin (to determine glucose and insulin concentrations) or EDTA (to determine amino acid concentrations and glucose enrichment). Samples were placed in ice and plasma was separated by centrifugation within 30 min of collection and then stored at -80 °C until final analyses. Muscle samples were rinsed in ice-cold saline immediately after collection, cleared of visible fat and connective tissue, frozen in liquid nitrogen and stored at -80 °C until final analysis.

Plasma glucose concentration was determined by using an automated glucose analyzer (Yellow Spring Instruments Co, Yellow Springs, OH). Plasma insulin concentration was measured by using a commercially available enzyme-linked immunosorbent assay (EMD Millipore, St Charles, MO).

The glucose tracer-to-tracee ratio (TTR) in plasma was determined by using gas-chromatography/mass-spectrometry (GC-MS, Hewlett-Packard MSD 5973 system with capillary column) after derivatizing glucose with acetic anhydride. Plasma leucine concentration was determined by using GC-MS (MSD 5973 System, Hewlett-Packard) after adding a known amount of nor-leucine to aliquots of each plasma sample and derivatization with t-butyldimethylsilyl (29). The concentrations of all other amino acids in plasma were determined by using the EZ:faast physiological (free) amino acid kit (Phenomenex, Torrence, CA) and GC-MS analysis per manufacturer instructions.
Western analysis was used to quantify the contents of p-AMPK$$^{\text{Thr172}}$$, p-ACC$$^{\text{Ser79}}$$, p-mTOR$$^{\text{Ser2448}}$$, p-p70S6K$$^{\text{Thr389}}$$, p-AKT$$^{\text{Ser473}}$$, p-AKT$$^{\text{Thr308}}$$, and GSKβ$$^{\text{Ser9}}$$ in muscle. Frozen muscle tissue was rapidly homogenized in ice-cold Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA) and proteins were extracted (30). Twenty to thirty µg of protein from each sample were loaded onto 7% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA), separated by SDS-PAGE, and transferred to Immobilon-FL polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The blotted membranes were incubated with the following primary antibodies: rabbit polyclonal anti-phospho-AMPKα (Thr172) antibody (#2531; Cell Signaling Technology), rabbit polyclonal anti-phospho-ACC (Ser79) antibody (#3661; Cell Signaling Technology), rabbit polyclonal anti-phospho-mTOR (Ser2448) antibody (#2971; Cell Signaling Technology), rabbit monoclonal anti-phospho-p70S6K (Thr389) antibody (#9234; Cell Signaling Technology), rabbit monoclonal anti-phospho-AKT (Ser473) antibody (#4060; Cell Signaling Technology), rabbit monoclonal anti-phospho-AKT (Thr308) antibody (#4056; Cell Signaling Technology), rabbit monoclonal anti-phospho-GSK-3β (Ser9) antibody (#9323; Cell Signaling Technology), and goat polyclonal anti-ACTIN antibody (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA). p-AKT$$^{\text{Ser473}}$$ and ACTIN blots were incubated with LI-COR IRDye 800-labeled secondary antibodies (926-32213 and 926-32214, respectively; LI-COR Biosciences, Lincoln, NE) and developed by using the Odyssey® Infrared Imaging System (LI-COR Bioscience). p-AMPK$$^{\text{Thr172}}$$, p-ACC$$^{\text{Ser79}}$$, p-mTOR$$^{\text{Ser2448}}$$, p-p70S6K$$^{\text{Thr389}}$$, p-AKT$$^{\text{Thr308}}$$ and p-GSKβ$$^{\text{Ser9}}$$ blots were incubated with a horseradish peroxidase-conjugated secondary antibody (#7074; Cell Signaling Technology) and developed by using the Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences, Piscataway, NJ). The contents of p-AMPK$$^{\text{Thr172}}$$, p-ACC$$^{\text{Ser79}}$$, p-mTOR$$^{\text{Ser2448}}$$, p-p70S6K$$^{\text{Thr389}}$$, p-AKT$$^{\text{Ser473}}$$, p-AKT$$^{\text{Thr308}}$$, and p-
GSKβSer\(^{9}\) were expressed relative to a single sample loading control and relative to ACTIN. The results were the same, irrespective of the control protein used.

**Calculations**

Endogenous glucose rate of appearance (Ra) in plasma in the basal state was calculated by dividing the glucose tracer infusion rate by the average plasma glucose TTR during the last 20 min of the basal period. During the clamp, total glucose Ra was calculated by dividing the glucose tracer infusion rate by the average plasma glucose TTR during the last 20 min of the clamp procedure and adding the tracer infusion rate to this value. Total glucose Ra represents the sum of endogenous glucose production plus the rate of infused glucose (dextrose plus tracer) and equals the rate of glucose disappearance (Rd) from plasma. Endogenous glucose Ra during the clamp was therefore calculated by subtracting the glucose infusion rate (dextrose plus tracer) from total glucose Ra. Leg glucose uptake was calculated as the product of leg plasma flow [i.e., blood flow × (1 - hematocrit)] and the plasma glucose arterio-venous concentration difference.

**Statistical analyses**

Statistical analyses were carried out with SPSS version 21 for Windows (IBM, Armonk, NY). All data sets were tested for normality by using the Kolmogorov-Smirnov test and skewed data sets were log-transformed for further analysis. Student’s t-test was used to compare basic characteristics of subjects in the whey protein and leucine groups. Three-way (group [whey protein vs leucine] × study [control vs whey protein or leucine ingestion] × condition [basal vs clamp]) repeated measures analysis of variance (ANOVA) and Tukey’s post-hoc procedure were used to evaluate the effects of whey protein and leucine ingestion on plasma glucose, amino acid
and insulin concentrations, leg plasma flow, whole-body and leg glucose kinetics and p-AMPK<sup>Thr172</sup>, p-ACC<sup>Ser79</sup>, p-mTOR<sup>Ser2448</sup>, p-p70S6K<sup>Thr389</sup>, p-AKT<sup>Ser473</sup>, p-AKT<sup>Thr308</sup>, and p-GSKβ<sup>Ser9</sup> contents in muscle. In addition, analysis of covariance (ANCOVA) with plasma insulin concentration as a covariate was used to compare the effects of whey protein and leucine ingestion on whole-body and leg glucose kinetics to account for small but potentially important differences in insulin concentration between studies. The glucose Rd-to-insulin ratio was compared by using two-way (group [whey protein vs leucine] × study [control vs whey protein or leucine ingestion]) ANOVA and Tukey’s post-hoc procedure.

A P-value of ≤0.05 was considered statistically significant. Unless otherwise noted, data are presented as mean ± SEM or median [quartiles] for normally distributed and skewed data sets, respectively.

RESULTS

Arterial plasma glucose, insulin, and amino acid concentrations (Table 2)

Basal arterial plasma glucose, insulin and amino acid concentrations were not different between the whey protein and leucine ingestion groups and between the whey protein or leucine ingestion and respective control studies within each group. During the clamp procedure, glucose concentration was maintained at the 5.6 mM target (mean: 5.62 ± 0.02 mM) in all studies. Insulin concentration increased ~10-fold in all studies, and the clamp-induced increase was ~10% greater (P < 0.01) during whey and leucine ingestion than during their respective control studies. Leucine concentration decreased by ~40% during insulin infusion in the control studies and increased to ~70% above basal values during whey protein and leucine ingestion. Total branched-chain amino acid concentration decreased by ~40% during insulin infusion in the
control studies and increased by ~60% above basal values during whey protein ingestion, but did
not change during leucine ingestion. Total amino acid, essential amino acid and non-essential
amino acid concentrations decreased during insulin infusion in the control studies and during
leucine ingestion, but increased during whey protein ingestion. Accordingly, total amino acid,
branched-chain amino acid, essential amino acid and non-essential amino acid concentrations
during insulin infusion were 25-50% lower (P < 0.01) during leucine ingestion than whey protein
ingestion.

Whole-body glucose kinetics

Basal endogenous glucose Ra was not different between the control and whey protein
ingestion studies (837 ± 41 and 809 ± 38 µmol·min⁻¹, respectively) or the control and leucine
ingestion studies (773 ± 37 and 772 ± 26 µmol·min⁻¹, respectively). During the clamp, endogenous glucose Ra was almost completely (by 90.5 ± 1.3 %) suppressed in all studies (to 77 ± 24 and 65 ± 14 µmol·min⁻¹ in the control and whey protein ingestion studies, respectively and
to 69 ± 22 and 93 ± 25 µmol·min⁻¹ in the control and leucine ingestion studies respectively; main
effect of clamp, P < 0.001; no significant interactions and no significant main effect of group).

Glucose Rd during the clamp was significantly lower during whey protein ingestion than during
the control drink ingestion (P < 0.01), whereas leucine ingestion had no effect on glucose Rd
during the clamp (Figure 1). The difference in the effect of whey protein and leucine ingestion
on glucose Rd during the clamp persisted even when the small differences in plasma insulin
concentration among studies were taken into account by using ANCOVA or by evaluating the
glucose Rd-to-insulin ratio, which was 38.8 [30.8, 61.8] and 51.9 [41.0, 77.3] µmol·µU min⁻¹·ml⁻¹
in the whey protein and corresponding control studies, respectively (P < 0.01) and 52.3 [43.3,
and 52.3 [43.9, 73.2] µmol·µU·min⁻¹·ml⁻¹ in the leucine and corresponding control studies, respectively.

Leg plasma flow and glucose kinetics

Basal leg plasma flow was not different between the whey protein and leucine ingestion and respective control studies (Table 2). During the clamp, leg plasma flow significantly increased (compared to basal conditions) in all studies (main effect of clamp, P < 0.001) and neither whey protein nor leucine ingestion affected the clamp-induced increase (Table 2).

The basal rate of leg glucose uptake was very small and not different between the whey protein or leucine ingestion studies and their respective control studies. During the clamp, leg glucose uptake increased by more than 10-fold in all studies (P < 0.001); the clamp-induced increase was reduced (by ~20%) with whey protein (P < 0.05) but not leucine ingestion (Figure 1).

Phosphorylation of signaling transduction proteins in muscle (Figures 2 and 3)

Basal p-AMPKThr172, p-ACCSer79, p-mTORSer2448, p-p70S6KThr389, p-AKTSer473, and p-AKTThr308, and p-GSKβSer9 (data not shown) contents in muscle were not different between groups and studies (whey protein or leucine ingestion and respective controls). During the clamp procedure, p-mTORSer2448, p-p70S6KThr389, p-AKTSer473, and p-AKTThr308 increased in all studies (P < 0.001), whereas p-AMPKThr172, p-ACCSer79, and GSKβSer9 (data not shown) were unchanged compared with basal values. The clamp-induced increases in p-mTORSer2448 and p-p70S6KThr389 were greater during whey protein and leucine ingestion relative to their respective control studies but not different during whey protein and leucine ingestion. The clamp-induced increases in p-
AKT^{Ser473} and p-AKT^{Thr308} were not affected by whey protein or leucine ingestion and neither whey protein nor leucine ingestion had an effect on p-GSKβ^{Ser9} (data not shown).

**DISCUSSION**

In the present study, we tested the hypothesis that protein ingestion impairs insulin-stimulated glucose disposal via leucine-mediated skeletal muscle mTOR-p70S6K phosphorylation and subsequent inhibition of the insulin signaling cascade. Accordingly, we evaluated rates of whole-body and leg glucose disposal and p-mTOR^{Ser2448}, p-p70S6K^{Thr389}, p-AKT^{Ser473} and p-AKT^{Thr308} in skeletal muscle during basal conditions and during a hyperinsulinemic-euglycemic clamp procedure with and without concomitant whey protein or leucine ingestion. Ingestion of whey protein and leucine alone (which matched the amount of leucine present in whey protein) caused the same increase in plasma leucine concentration and muscle p-mTOR^{Ser2448} and p-p70S6K^{Thr389} contents, but did not affect muscle p-AKT^{Ser473} and p-AKT^{Thr308}. Moreover, whey protein, but not leucine, ingestion impaired glucose disposal. These results indicate that protein ingestion could be an important regulator of postprandial glucose homeostasis but the adverse effect of protein ingestion on glucose disposal is not mediated by leucine and occurs independently of mTOR and AKT signaling in muscle.

The results from several studies conducted in cultured myotubes and isolated rat skeletal muscles suggest that leucine-mediated mTOR signaling has adverse effects on insulin sensitivity because they demonstrate that leucine stimulates mTOR and IRS serine phosphorylation (15; 16) and impairs PI3K-AKT signaling and insulin-mediated glucose uptake (15; 16; 31). In addition, it has been demonstrated that treatment with rapamycin, an mTOR inhibitor, abolishes the adverse effect of hyperaminoacidemia on insulin-mediated glucose disposal both *in vivo*
people and *in vitro* in cultured myocytes (16; 20). However, rapamycin does not directly inhibit mTOR kinase activity and has mTOR-independent effects throughout the body (32-34), which confounds the interpretation of those results. The data from our study suggest that mTOR-p70S6K signaling is not involved in protein-induced inhibition of glucose uptake during hyperinsulinemia in people. We found that both whey protein and leucine ingestion increased p-mTOR\textsuperscript{Ser2448} and p-p70S6K\textsuperscript{Thr389} in muscle without affecting p-AKT\textsuperscript{Ser473} and p-AKT\textsuperscript{Thr308}. Moreover, whey protein, but not leucine, reduced glucose uptake in the absence of changes in skeletal muscle p-AKT\textsuperscript{Ser473} and p-AKT\textsuperscript{Thr308} contents. In concert with our findings, data from previous studies conducted in human subjects (23; 35) illustrate that a ~6 h infusion of an amino acid mixture (~80 g amino acids containing ~7 g leucine) and a 2 h infusion of ~2 g of leucine during hyperinsulinemia-euglycemia increased S6K activity and p70S6K and IRS serine phosphorylation and impaired glucose uptake, without a decrease in AKT phosphorylation. Together these data suggest that the cellular mechanism responsible for the adverse effect of hyperaminoacidemia on glucose disposal lies downstream of AKT or occurs independently of the inhibitory mTOR-p70S6K-IRS signaling pathway to AKT. It is possible that protein ingestion interfered with non-insulin mediated glucose disposal, which accounts for 15-25 % of total glucose disposal during hyperinsulinemic-euglycemic clamp conditions (36; 37).

The mechanism(s) responsible for the effect of both whey protein and leucine ingestion on mTOR phosphorylation are unclear. The results from studies conducted in cultured rat muscles suggest that both glucose and leucine stimulate mTOR phosphorylation through downregulation of AMPK (7; 31; 38; 39). However, we found that insulin-glucose infusion alone and the ingestion of whey protein and leucine increased muscle p-mTOR\textsuperscript{Ser2448} without an effect on muscle p-AMPK\textsuperscript{Thr172} content or its downstream target p-ACC\textsuperscript{Ser79}. This dissociation between
AMPK and mTOR signaling is consistent with the results from several previous studies conducted in human subjects during glucose-insulin infusion (40-43), and suggests that other mechanisms (e.g., Vps34 or phosphatidic acid signaling (44-47)) were responsible for both the insulin-glucose and whey protein and leucine-mediated increases in muscle p-mTOR<sup>Ser2448</sup> in our study.

The insulin-mediated suppression of endogenous glucose Ra was not affected by whey protein or leucine ingestion in our study, most likely because endogenous glucose production is very sensitive to insulin (48) and was almost completely suppressed by the hyperinsulinemia achieved in our study. However, it is also possible that the amount of protein given in our study (~23 g) was not enough to elicit an effect. Intravenous administration of amino acids in excess of ~80 g was found to blunt the insulin-mediated suppression of endogenous glucose production both during low- and high-dose insulin infusion (21; 23; 49) whereas administration of ~12 g had no effect (22).

Our study has some limitations that need to be considered. First, we studied only 50-65 y old obese postmenopausal women. Although it is possible that the findings from our study cannot be extrapolated to other populations, we believe this is unlikely because amino acid-induced insulin resistance has previously been observed in non-obese men and women across a wide age range (i.e., 18-70 y) (21-23). Secondly, it is possible that our study did not contain an adequate number of subjects to detect a leucine-mediated decrease in whole-body glucose Rd and leg glucose uptake. However, this seems unlikely because mean whole-body glucose Rd and leg glucose uptake during the clamp were nearly identical after leucine and control drink ingestion and the individual values were numerically greater after leucine than control drink ingestion in 6 and 8 out of the 11 subjects, respectively.
In summary, we found that whey protein ingestion impairs glucose disposal during hyperinsulinemia, both at the whole-body level and across the leg, independent of leucine-mediated mTOR-p70S6K and AKT signaling. Protein intake could therefore be an important regulator of postprandial glucose clearance. Additional studies are needed to determine the precise mechanism(s) responsible for the adverse effect of protein ingestion on glucose disposal, the extent to which it may be counterbalanced by the stimulatory effect of protein ingestion on insulin secretion (8; 50), and how long-term changes in dietary protein intake affect glucose homeostasis.
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DUALITY OF INTEREST

None of the authors had any potential conflicts of interest. No funding entity had any role in the design, implementation, analysis, or interpretation of the data.
AUTHOR CONTRIBUTIONS

GIS: conducting the studies, processing study samples, collecting data, performing data analyses, and writing the manuscript. JY: processing study samples, collecting data, assisting with data analysis and editing the manuscript. KS: processing study samples and editing the manuscript. SJK, FM and DNR: assisting in conducting the studies and editing the manuscript. SK: designing the studies, obtaining funding for the studies, medical supervision of the studies and editing the manuscript. BM: designing the studies, obtaining funding for the studies, involved in conducting the studies, processing study samples, collecting data, performing the final data analyses, and writing the manuscript. BM is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

PRIOR PRESENTATION

Preliminary results from this study were presented in abstract form at the 2014 Experimental Biology meeting in San Diego, CA, 26 – 30 April 2014.
REFERENCES


Table 1. Subjects’ body composition and basic metabolic characteristics.

<table>
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<tr>
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<th>Whey protein group (n = 11)</th>
<th>Leucine group (n = 11)</th>
<th>P-value*</th>
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<tbody>
<tr>
<td>Body mass index (kg/m(^2))</td>
<td>33.6 ± 0.8</td>
<td>36.0 ± 1.5</td>
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<td>Body mass (kg)</td>
<td>90.3 ± 2.3</td>
<td>96.4 ± 4.5</td>
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<td>Body fat (%)</td>
<td>48.1 ± 1.0</td>
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<td>Subcutaneous abdominal fat (cm(^3))</td>
<td>2,940 ± 118</td>
<td>3,197 ± 232</td>
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<td>Intra-abdominal fat (cm(^3))</td>
<td>1,431 ± 137</td>
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<td>Fat free mass (kg)</td>
<td>46.8 ± 1.4</td>
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<td>Leg lean mass (kg)</td>
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<td>15.6 ± 0.6</td>
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<td><strong>Plasma concentrations</strong></td>
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<td>Glucose (mM)†</td>
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<td>5.43 ± 0.17</td>
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<td>Glucose - 2 h post OGTT (mM)</td>
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<td>Triglycerides (mM)†</td>
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<td>1.27 ± 0.20</td>
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</tr>
<tr>
<td>Total cholesterol (mM)†</td>
<td>6.10 ± 0.30</td>
<td>5.12 ± 0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>HDL-cholesterol (mM)†</td>
<td>1.49 ± 0.09</td>
<td>1.51 ± 0.10</td>
<td>0.87</td>
</tr>
<tr>
<td>LDL-cholesterol (mM)†</td>
<td>3.75 ± 0.27</td>
<td>3.02 ± 0.13</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. OGTT: oral glucose tolerance test. * Values were determined by using Student’s t-test for independent samples. † Values were obtained after an overnight fast.
Table 2. Effects of whey protein and leucine ingestion on arterial plasma amino acid and insulin concentrations, arterio-venous plasma glucose concentration differences, and leg plasma flow.

<table>
<thead>
<tr>
<th></th>
<th>Whey protein group</th>
<th>Leucine group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Whey protein</td>
</tr>
<tr>
<td>Leucine (µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>113 ± 4</td>
<td>120 ± 3</td>
</tr>
<tr>
<td>Clamp</td>
<td>68 ± 5*</td>
<td>205 ± 10*†</td>
</tr>
<tr>
<td>Branched-chain amino acids (µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>368 ± 18</td>
<td>398 ± 12</td>
</tr>
<tr>
<td>Clamp</td>
<td>239 ± 21*</td>
<td>636 ± 30*†</td>
</tr>
<tr>
<td>Essential amino acids (µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>807 ± 38</td>
<td>864 ± 33</td>
</tr>
<tr>
<td>Clamp</td>
<td>571 ± 38*</td>
<td>1,385 ± 54*†</td>
</tr>
<tr>
<td>Non-essential amino acids (µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>903 ± 40</td>
<td>931 ± 46</td>
</tr>
<tr>
<td>Clamp</td>
<td>713 ± 32*</td>
<td>1,043 ± 39*†</td>
</tr>
<tr>
<td>Total amino acids (µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1,711 ± 75</td>
<td>1,795 ± 75</td>
</tr>
<tr>
<td>Clamp</td>
<td>1,284 ± 66*</td>
<td>2,428 ± 88*†</td>
</tr>
<tr>
<td>Insulin (µU·ml⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Clamp</td>
<td>53 ± 2*</td>
<td>61 ± 4*†</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal - Artery</td>
<td>5.09 ± 0.05</td>
<td>5.12 ± 0.06</td>
</tr>
<tr>
<td>Clamp - Artery</td>
<td>5.61 ± 0.04§</td>
<td>5.60 ± 0.06§</td>
</tr>
<tr>
<td>Basal - Vein</td>
<td>5.00 ± 0.05</td>
<td>5.04 ± 0.06</td>
</tr>
<tr>
<td>Clamp - Vein</td>
<td>4.42 ± 0.16§</td>
<td>4.59 ± 0.10§</td>
</tr>
<tr>
<td>A-V glucose difference (mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.09 ± 0.02</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Clamp</td>
<td>1.19 ± 0.16*</td>
<td>1.01 ± 0.14*†</td>
</tr>
<tr>
<td>Plasma flow (ml·min⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>173 ± 25</td>
<td>178 ± 23</td>
</tr>
<tr>
<td>Clamp</td>
<td>219 ± 31§</td>
<td>224 ± 31§</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. A-V: Arterio-venous. Three-way ANOVA revealed a significant study interaction (P < 0.001) for branched-chain, essential, non-essential and
total amino acid concentrations, a significant study × condition interaction (P < 0.001) for leucine and insulin concentrations, and a significant main effect of clamp (P < 0.01) for glucose concentrations and plasma flow. ANCOVA with plasma insulin concentration as a covariate revealed a significant study [control vs whey protein] × condition [basal vs clamp]) interaction (P < 0.05) for the arterio-venous glucose concentration difference. Tukey’s post-hoc analysis revealed the following significant differences: * Value significantly different from corresponding basal value (P < 0.01). † Value significantly different from corresponding control values (P < 0.01). ‡ Value significantly different from corresponding value in the whey protein group (P < 0.01). § Significant main effect of clamp (P < 0.001).
FIGURE LEGENDS

Figure 1. Effects of whey protein and leucine ingestion on whole-body glucose rate of disappearance and leg glucose uptake (white bars = basal; black bars = clamp). Data are mean ± SEM. Three-way ANOVA revealed a significant group [whey protein vs leucine groups] × study [control vs whey protein or leucine ingestion] × condition [basal vs clamp]) interaction (P < 0.001) for whole-body glucose rate of disappearance. ANCOVA with plasma insulin concentration as a covariate revealed a significant study [control vs whey protein] × condition [basal vs clamp]) interaction (P < 0.05) for whole-body glucose rate of disappearance and leg glucose uptake. Tukey’s post-hoc analysis revealed the following significant differences: * value significantly different from corresponding basal value (P < 0.01); † value significantly different from corresponding control value (P < 0.01); ‡ value significantly different from corresponding control value (P < 0.05).

Figure 2. Effect of whey protein ingestion on p-AMPK\textsuperscript{Thr172}, p-ACC\textsuperscript{Ser79}, p-mTOR\textsuperscript{Ser2448}, p-p70S6K\textsuperscript{Thr389}, p-AKT\textsuperscript{Ser473} and p-AKT\textsuperscript{Thr308} (arbitrary units) in muscle (white bars = basal; black bars = clamp). AMPK, p-ACC, mTOR, and p-p70S6K data are mean ± SEM; AKT data were log-transformed for ANOVA and are presented as backtransformed geometric means and errors. Three-way ANOVA revealed a significant study [control vs whey protein or leucine ingestion] × condition [basal vs clamp]) interaction (P < 0.05) for p-mTOR\textsuperscript{Ser2448} and p-p70S6K\textsuperscript{Thr389} and a significant main effect of clamp (P < 0.001) for p-AKT\textsuperscript{Ser473} and p-AKT\textsuperscript{Thr308}. Tukey’s post-hoc analysis revealed the following significant differences: * Value significantly different from corresponding basal value (P < 0.05); † value significantly different from corresponding control value (P < 0.05).
Figure 3. Effect of leucine ingestion on p-AMPK\textsuperscript{Thr172}, p-ACC\textsuperscript{Ser79}, p-mTOR\textsuperscript{Ser2448}, p-p70S6K\textsuperscript{Thr389}, p-AKT\textsuperscript{Ser473} and p-AKT\textsuperscript{Thr308} (arbitrary units) in muscle (white bars = basal; black bars = clamp). AMPK, p-ACC, mTOR, and p-p70S6K data are mean ± SEM; AKT data were log-transformed for ANOVA and are presented as backtransformed geometric means and errors. Three-way ANOVA revealed a significant study [control vs whey protein or leucine ingestion] × condition [basal vs clamp]) interaction (P < 0.05) for p-mTOR\textsuperscript{Ser2448} and p-p70S6K\textsuperscript{Thr389} and a significant main effect of clamp (P < 0.001) for p-AKT\textsuperscript{Ser473} and p-AKT\textsuperscript{Thr308}. Tukey’s post-hoc analysis revealed the following significant differences: * Value significantly different from corresponding basal value (P < 0.05); † value significantly different from corresponding control value (P < 0.05).
Figure 1. Effects of whey protein and leucine ingestion on insulin-stimulated whole-body glucose rate of disappearance and leg glucose uptake (white bars = basal; black bars = clamp). Data are mean ± SEM. Three-way ANOVA revealed a significant group [whey protein vs leucine groups] × study [control vs whey protein or leucine ingestion] × condition [basal vs clamp] interaction (P < 0.001) for whole-body glucose rate of disappearance. ANCOVA with plasma insulin concentration as a covariate revealed a significant study [control vs whey protein] × condition [basal vs clamp] interaction (P < 0.05) for whole-body glucose rate of disappearance and leg glucose uptake. Tukey’s post-hoc analysis revealed the following significant differences: * value significantly different from corresponding basal value (P < 0.01); † value significantly different from corresponding control value (P < 0.01); ‡ value significantly different from corresponding control value (P < 0.05).
Figure 2. Effect of whey protein ingestion on p-AMPK\textsuperscript{Thr172}, p-ACC\textsuperscript{Ser79}, p-mTOR\textsuperscript{Ser2448}, p-p70S6K\textsuperscript{Thr389}, p-AKT\textsuperscript{Ser473}, and p-AKT\textsuperscript{Thr308} (arbitrary units) in muscle (white bars = basal; black bars = clamp). AMPK, p-ACC, mTOR, and p-p70S6K data are mean ± SEM; AKT data were log-transformed for ANOVA and are presented as backtransformed geometric means and errors. Three-way ANOVA revealed a significant study [control vs whey protein or leucine ingestion] × condition [basal vs clamp]) interaction (P < 0.05) for p-mTOR\textsuperscript{Ser2448} and p-p70S6K\textsuperscript{Thr389} and a significant main effect of clamp (P < 0.001) for p-AKT\textsuperscript{Ser473} and p-AKT\textsuperscript{Thr308}. Tukey’s post-hoc analysis revealed the following significant differences: * Value significantly different from corresponding basal value (P < 0.05); † value significantly different from corresponding control value (P < 0.05).
Figure 3. Effect of leucine ingestion on p-AMPK<sub>Thr172</sub>, p-ACC<sub>Ser79</sub>, p-mTOR<sub>Ser2448</sub>, p-p70S6K<sub>Thr389</sub>, p-AKT<sub>Ser473</sub> and p-AKT<sub>Thr308</sub> (arbitrary units) in muscle (white bars = basal; black bars = clamp). AMPK, p-ACC, mTOR, and p-p70S6K data are mean ± SEM; AKT data were log-transformed for ANOVA and are presented as backtransformed geometric means and errors. Three-way ANOVA revealed a significant study [control vs whey protein or leucine ingestion] × condition [basal vs clamp]) interaction (P < 0.05) for p-mTOR<sub>Ser2448</sub> and p-p70S6K<sub>Thr389</sub> and a significant main effect of clamp (P < 0.001) for p-AKT<sub>Ser473</sub> and p-AKT<sub>Thr308</sub>. Tukey’s post-hoc analysis revealed the following significant differences: * Value significantly different from corresponding basal value (P < 0.05); † value significantly different from corresponding control value (P < 0.05).